AD NUMBER

AD481044

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; Feb 1966. Other requests shall be referred to U.S. Army Biological Labs., Fort Detrick, MD.

AUTHORITY

BORL D/A ltr, 28 Sep 1971

AD

481044

TECHNICAL MANUSCRIPT 268

ELECTRON MICROSCOPIC AND IMMUNOFLUORESCENT STUDY OF CHIKUNGUNYA VIRUS INFECTION IN TISSUE CULTURE

John D. White

Lazarus L. Kupferberg

FEBRUARY 1966

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

DDC AVAILABILITY NOTICES

Qualified requestors may obtain copies of this publication from DDC.

Foreign announcement and dissemination of this publication by DDC is not authorized.

Release or announcement to the public is not authorized.

DISPOSITION INSTRUCTIONS

Destroy this publication when it is no longer needed. Do not return it to the originator.

The findings in this publication are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 268

ELECTRON MICROSCOPIC AND IMMUNOFLUORESCENT STUDY OF CHIKUNGUNYA VIRUS INFECTION IN TISSUE CULTURE

John D. White

Lazarus L. Kupferberg

Pathology Division
DIRECTORATE OF MEDICAL RESEARCH

Project 1C014501B71A01

February 1966

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

ABSTRACT

The development of chikungunya virus was investigated by immunofluorescent staining and electron microscopic observations. The virus was propagated in hamster kidney cells and viral antigen was first detected by immunofluorescence at 8 hours. The first alteration in subcellular structure, however, was observed at 12 hours. At that time electron-dense particles were seen surrounding cytoplasmic vacuoles. Mature virus particles were seen only within cytoplasmic vacuoles or in juxtaposition to the external surface of the cytoplasmic membrane. These particles were similar morphologically to particles seen in partially purified brain suspension from infected suckling mice. The results are compared with those reported in the literature for other arboviruses.

ELECTRON MICROSCOPIC AND IMMUNOFLUORESCENT STUDY OF CHIKUNGUNYA VIRUS INFECTION IN TISSUE CULTURE

Recent reports in the literature have described the intracellular development of various arboviruses. These viruses have been studied in ultrathin sections of tissue cultures and certain similarities have been noted. In the course of our examination of ultrathin sections it was noted that chikungunya virus also resembled the morphologic descriptions reported in the literature for certain of the arboviruses, namely Mayaro virus,[1] VEE[2] and WEE.[3] These viruses, related serologically, are classified as group A arboviruses. More recently, Ota[4] described the ultrastructure of Japanese B encephalitis virus (group B) in tissue culture and noted the similarity to the aforementioned viruses.

This report describes the ultrastructural changes in hamster kidney cells infected with chikungunya virus. These changes are interpreted in comparison with other arboviruses.

A line of hamster kidney cells (HKL)* was grown in medium 199 supplemented with 10% calf serum, vitamins, and glutamine at 35.5 C. Cultures of cells in Leighton tubes were washed twice with phosphate-buffered saline (PBS) and covered with 1.0 ml of heart infusion broth (HIB) containing 10^6 $MICLD_{50}$ of virus. After absorption the cultures were washed twice with PBS and fresh medium was added. Cultures were harvested at various times after infection and processed for immunofluorescent staining and electron microscopy.

The immunofluorescent reagent was prepared from sera collected from monkeys two weeks after the injection of 10^6 $MICLD_{50}$ of chikungunya virus. The direct staining procedure was used throughout. Preimmunization sera were not reactive in immunofluorescent tests.

Material for electron microscopy was fixed in 1% osmium tetroxide in Millonig's buffer, dehydrated, embedded in Epon 812, sectioned on a Servall MT2 ultra-microtome, stained with uranyl acetate, and viewed in a RCA EMU-3G microscope.

At 2, 4, 6, 8, 10, 12, 15, 18, and 24 hours after infection, cells were stained with the fluorescein-labeled antibody solution and examined. Viral antigen was first seen in cells harvested at 8 hours. Infected cells were few in number at this time. The appearance of these cells indicated that viral antigen was concentrated at one locus in the perinuclear region of the cytoplasm (Figure 1). Subsequently there was no apparent increase in numbers of infected cells. No attempt was made to quantitate this observation, which was based on visual comparison of stained material. The appearance of infected cells seen after 8 hours was quite similar. The fluorescence was limited to a thin peripheral band

* It has been subsequently learned that this line of HKL cells is in fact mouse L cells. Personal communication, Dr. J.E. Officer, Virus and Rickettsia Division.

(Figure 2) when the cells were studied in median optical section. A surface view confirmed the peripheral location in that fluorescence was uniform throughout the cell. In a few cells (Figure 3) a perinuclear inclusion was still apparent. Fluorescent viral antigen was never seen in the nucleus.

Cells for electron microscopy were obtained at 6, 9, 12, 15, and 18 hours after infection. HKL cells from noninfected cultures were also observed throughout the experiment. During this time no degenerative changes were noted in the ultrastructure of noninfected cells. The nucleus was round with prominent nucleoli. The cytoplasmic organelles appeared normal (Figure 4).

The earliest change seen in cells from infected cultures was a dilatation of the rough membraned vesicles (endoplasmic reticulum). This imparted a vesicular appearance to the cytoplasm (Figure 5). At higher magnification (Figure 6) electron-dense particles larger than the ribosomes were seen in the cytoplasm near vesicles. Figure 7 shows cytoplasm of a cell 15 hours after infection. The perivacuolar location of these electron-dense particles was prominent. The particles, approximately 30 mμ in diameter, were fairly uniform in density and size, and did not have a limiting membrane. Some particles were free in the cytoplasm and not associated with cellular membranes. In some cells these latter particles were arranged in loosely packed aggregates. One of the largest aggregates, shown in Figure 8 was 700 mμ in diameter. The individual particles in the aggregates were 30 mμ in diameter. All cytoplasmic particles were devoid of a limiting membrane. Particles with a limiting membrane were seen only in vacuoles (Figure 9) or external to the cytoplasmic membrane (Figure 10). Figure 11 illustrates a group of viral particles apparently emerging from the cell. Note that at least one of the particles within the bounds of the cell does not possess a membrane. Membrane-limited particles consisted of a dense core, 30 mμ in diameter, surrounded by an electron-transparent area of 10 to 15 mμ. The diameter of the virus is approximately 60 mμ. Similar particles were seen in grids prepared with partially purified suspensions of infected suckling mouse brain.

An attractive hypothesis for the intracellular development of chikungunya virus would propose that viral nucleic acid is first formed in association with the ribosomes of the rough endoplasmic reticulum. A dense particle, the virus precursor, is formed around cytoplasmic vacuoles. These particles acquire a peripheral coat either after crossing a membrane into a vacuole or after migration through the cytoplasm and emergence from the cell. In either instance viral coat is formed after a cellular membrane is crossed. The immunofluorescent data in conjunction with electron microscopy tend to support this hypothesis. Viral fluorescence, when first seen, was perinuclear and the dilatation of endoplasmic reticulum seen in the electron microscope tended to be polar. At subsequent stages immunofluorescence was peripheral. A greater proportion of mature virus-like particles were seen by electron microscopy at the periphery of cells rather than within cytoplasmic compartments.

The ultrastructural changes seen in HKL cells infected with chikungunya virus are remarkably similar to those reported by Morgan, Howe, and Rose for WEE infection of tissue cultures[3] and of Mussgay and Weibel for VEE.[2] Mayaro virus described by Saturno[1] and Japanese B encephalitis virus (JBE) described by Ota[4] are also morphologically similar. In the developmental cycle of these viruses, dense particles enter vacuoles and acquire a limiting membrane rather than emerging from the cells to mature. Yellow fever virus, a group B arbovirus, is similar morphologically to these viruses. In our laboratories we have seen mature yellow fever virus only in vacuoles.[5]

In conclusion, arboviruses can be classified into various groups on the basis of their serological relationships and dissimilarities. It is not surprising, therefore, that the relationships and dissimilarities of certain arboviruses are also reflected in their morphology and stages of development.

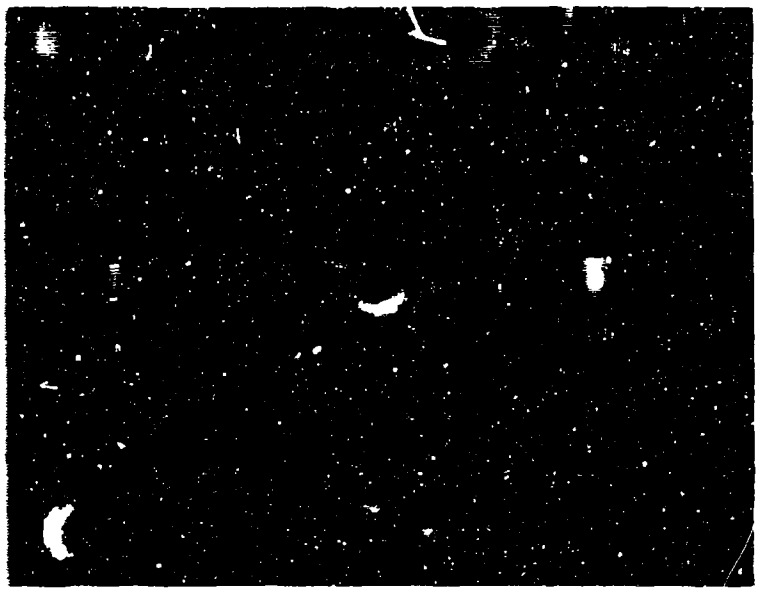


Figure 1. Immunofluorescent Staining of Infected Hamster Kidney Cells. Early appearance of immunofluorescence in cells stained at 8 hours. Note perinuclear aggregation of viral antigen. X 900.

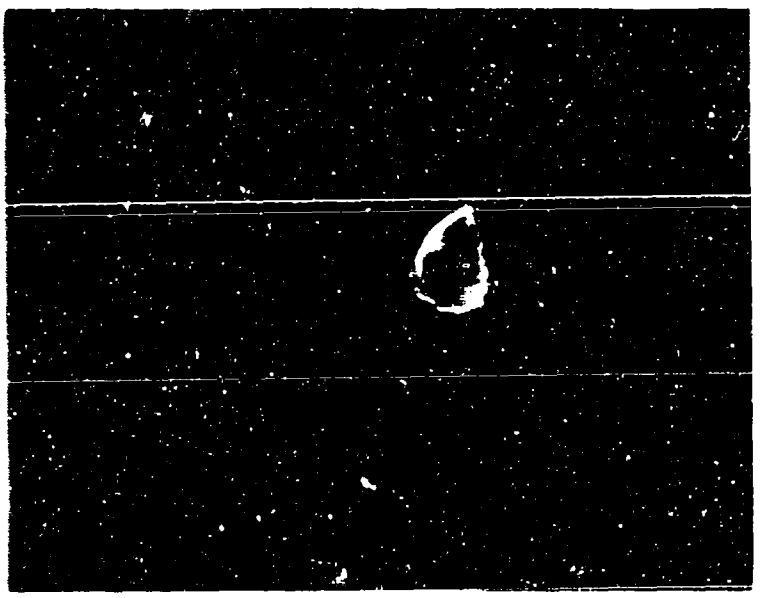


Figure 2. Immunofluorescent Staining of Infected Hamster Kidney Cells. Peripheral band of immunofluorescence is readily apparent in median optical section. Cell stained at 12 hours. X 900.

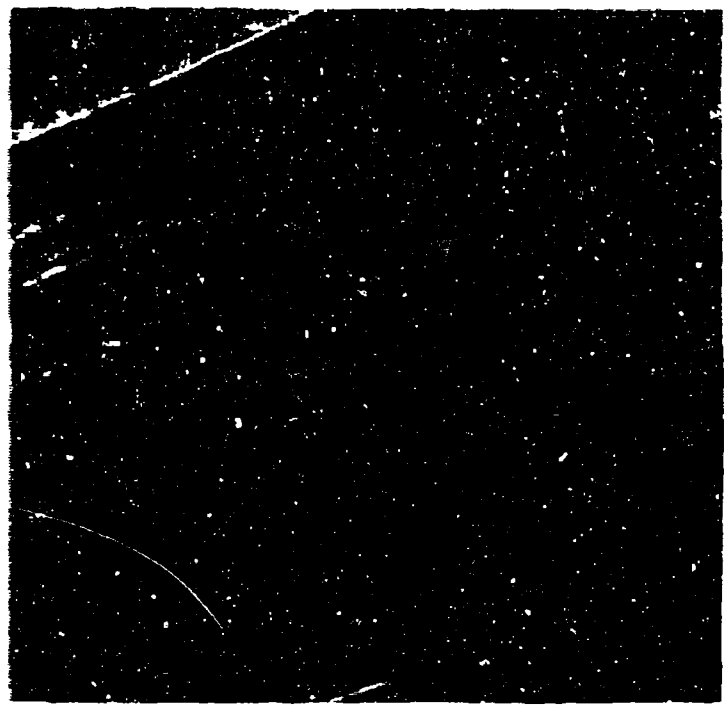


Figure 3. Electron Micrograph of Hamster Kidney Cells. Normal hamster kidney cell. X 7,500.

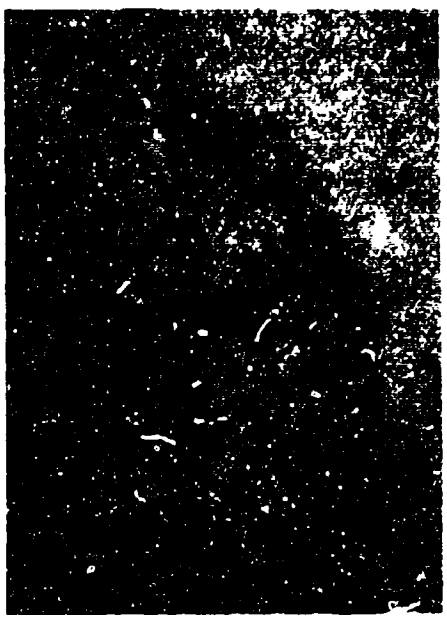
Figure 4. Electron Micrograph of Hamster Kidney Cell. Visicular appearance of cytoplasm (Cy) caused by dilation of rough-surfaced vesicles (endoplasmic reticulum) (En). There are no changes in the nucleus. Nine hours after infection. X 75,000.

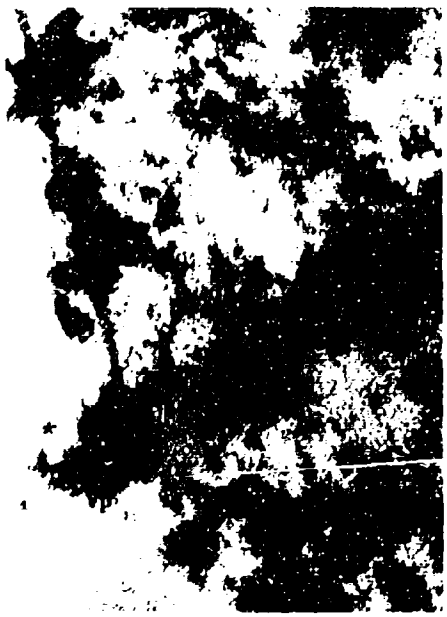
Figure 5. Electron Micrograph of Hamster Kidney Cells. Particles located amongst the ribosomes and in the adjacent cytoplasm. X 75,000.

Figure 6. Electron Micrograph of Hamster Kidney Cells. Two vacuoles surrounded by dense particles. There is no connection between particles nor attachment to the vacuole membrane. Some dense particles are seen in the cytoplasm at the right. X 100,000.

Figure 7. Electron Micrograph of Hamster Kidney Cells. Aggregate of dense particles in cytoplasm and mature virus external to the cytoplasmic membrane. X 25,000.

Figure 8. Electron Micrograph of Hamster Kidney Cells. Adjacent areas in the cytoplasm of an infected cell. Noce the large accumulation of dense particles in the cytoplasm. There are several membrane-limited virus particles (Vp) in the vacuole located in the lower portion. X 75,000.

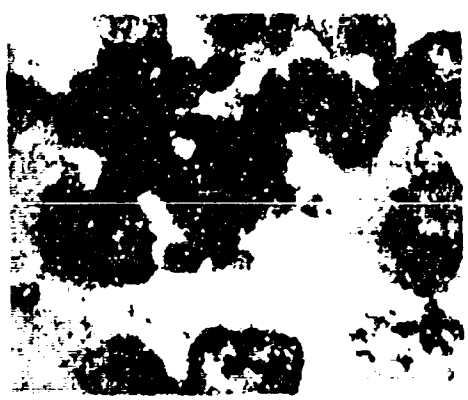




Figures 9 and 10. Electron Micrograph of Hamster Kidney Cells. These figures illustrate that the mature virus particle is found external to the cell and probably is formed from the electron-dense particles found in the cytoplasm. The mature virus consists of a dense core (25 mμ in diameter) surrounded by a zone of lesser density and a membrane 50 to 60 mμ in diameter.

Figure 9. (Left) At the Upper Portion of the Micrograph Mature Virus and Electron-Dense Particles are Located on Opposite Sides of the Cytoplasmic Membrane. X 50,000.

Figure 10. (Right) A Clump of Virus (*) Extrudes from the Cell and Three Dense-Particles are Immediately Adjacent in the Cytoplasm. X 75,000.



LITERATURE CITED

1. Saturno A. 1963. Morphology of Mayaro virus. Virology 21:131-133.

2. Musgay, M.; Weibel J. 1962. Electron microscopic and biological studies on the growth of Venezuelan equine encephalitis virus in KB cells. Virology 16:52-62.

3. Morgan, C.; Howe, C.; Rose, H.M. 1961. Structure and development of viruses as observed in the electron microscope: V. Western equine encephalomyelitis virus. J. Exp. Med. 113:219-233.

4. Ota, Z. 1965. Electron microscope study of the development of Japanese B. encephalitis virus in porcine kidney stable (PD) cells. Virology 25:372-378.

5. McGavran, M.H.; White, J.D. 1964. Electron microscopic and immunofluorescent observations on monkey liver and tissue culture cells infected with the Asibi strain of yellow fever virus. Amer. J. Pathol. 45:501-517.